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Possible involvement of pregnane X receptor–enhanced CYP24 expression in drug-induced osteomalacia

Jean Marc Pascussi,^{1,2} Agnes Robert,^{1,2} Minh Nguyen,³ Odile Walrant-Debray,³ Michèle Garabedian,³ Pascal Martin,⁴ Thierry Pineau,⁴ Jean Saric,⁵ Frédéric Navarro,⁶ Patrick Maurel,^{1,2} and Marie Josè Vilarem^{1,2}

¹INSERM 632, IFR122, Campus CNRS, Montpellier, France. ²Université Montpellier I, Montpellier, France. ³INSERM U561, Hôpital Saint-Vincent de Paul, Paris, France. ⁴Institut National de la Recherche Agronomique, Toulouse, France. ⁵Service de chirurgie digestive, Hôpital Saint-André, Bordeaux, France. ⁶Service de chirurgie digestive C, Hôpital Saint-Eloi, Montpellier, France.

Vitamin D controls calcium homeostasis and the development and maintenance of bones through vitamin D receptor activation. Prolonged therapy with rifampicin or phenobarbital has been shown to cause vitamin D deficiency or osteomalacia, particularly in patients with marginal vitamin D stores. However, the molecular mechanism of this process is unknown. Here we show that these drugs lead to the upregulation of 25-hydroxyvitamin D₃-24-hydroxylase (CYP24) gene expression through the activation of the nuclear receptor pregnane X receptor (PXR; NR1I2). CYP24 is a mitochondrial enzyme responsible for inactivating vitamin D metabolites. CYP24 mRNA is upregulated in vivo in mice by pregnenolone 16 α -carbonitrile and dexamethasone, 2 murine PXR agonists, and in vitro in human hepatocytes by rifampicin and hyperforin, 2 human PXR agonists. Moreover, rifampicin increased 24-hydroxylase activity in these cells, while, in vivo in mice, pregnenolone 16 α -carbonitrile increased the plasma concentration of 24,25-dihydroxyvitamin D₃. Transfection of PXR in human embryonic kidney cells resulted in rifampicin-mediated induction of CYP24 mRNA. Analysis of the human CYP24 promoter showed that PXR transactivates the sequence between –326 and –142. We demonstrated that PXR binds to and transactivates the 2 proximal vitamin D–responsive elements of the human CYP24 promoter. These data suggest that xenobiotics and drugs can modulate CYP24 gene expression and alter vitamin D₃ hormonal activity and calcium homeostasis through the activation of PXR.

Introduction

Vitamin D is essential for the maintenance of calcium homeostasis and for the development and maintenance of bones. Before acquiring its biological potential, vitamin D is transformed into 25-hydroxyvitamin D₃ [25(OH)D₃], the major form of vitamin D in the circulation, by two 25-hydroxylases (the mitochondrial CYP27A, and the microsomal CYP2R1) mainly expressed in the liver (1, 2). The 25(OH)D₃ is further hydroxylated in the 1 α position to form 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], by the CYP27B1 mainly expressed in the kidney (3). The active form of vitamin D₃, 1 α ,25(OH)₂D₃, elicits most of its biological effects by binding to a high-affinity receptor, the vitamin D receptor (VDR; NR1I1) (4). After binding 1 α ,25(OH)₂D₃, VDR forms heterodimers with the retinoid X receptor (RXR; NR2B1) and then binds to and transactivates the vitamin D–responsive elements (VDREs) present in the regulatory region of target genes (5). The classical VDREs consist of a direct repeat of nuclear receptor half-sites separated by 3 nucleotides (DR3) (6). In the classical vitamin D–responsive organs, including the intestine, bone, kidney, and parathyroid gland, vitamin D₃–activated VDR plays a central role in the regulation of calcium and phosphate homeostasis, bone mineralization,

inhibition of cell growth, and parathyroid hormone synthesis (3, 7). VDR is also expressed in many other nonclassical vitamin D–responsive organs, including the liver (8, 9).

The potent role of 1 α ,25(OH)₂D₃ in the regulation of serum calcium and phosphate levels requires a tight control of its blood concentration. This is reached through the balance between the rates of synthesis and degradation. CYP24 is a multifunctional 24-hydroxylase and the major catabolic enzyme of vitamin D (10). It directs the side-chain oxidation and cleavage of 1,25(OH)₂D and 25(OH)D to catabolic carboxylic acid end products. Notably, this enzyme converts 1 α ,25(OH)₂D₃ into 1 α ,24,25(OH)₃D₃, a product with decreased hormonal activity (3), and further into calcitroic acid, known as the final metabolite of 1 α ,25(OH)₂D₃ excreted in bile. Hypocalcemia increases the serum levels of 1 α ,25(OH)₂D₃ through stimulation of 1 α -hydroxylase (2) and suppresses the CYP24 activity through increased levels of parathyroid hormone (10). Inversely, hypercalcemia depresses 1 α -hydroxylase and increases the 24-hydroxylase activity, and high levels of 1 α ,25(OH)₂D₃ induce the 24-hydroxylase activity through VDR (11). The important role of the CYP24 gene has been further demonstrated in CYP24 knockout mice, which exhibit high ambient 1 α ,25(OH)₂D₃ levels and abnormal bone mineralization (12).

Osteomalacia is a metabolic bone disease, characterized by a defect of bone mineralization that can be caused by vitamin D deficiency or genetic alterations of vitamin D metabolism, vitamin D action, or other factors involved in the phosphocalcic metabolism. This pathology has also been suspected to result from the adverse effects of drugs. Some of the drugs most frequently associated with osteomalacia are phenobarbital, phenytoin, rifampicin,

Nonstandard abbreviations used: CHX, cycloheximide; Ct, cycle threshold; PXR, pregnane X receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D–responsive element.

Conflict of interest: The authors have declared that no conflict of interest exists.

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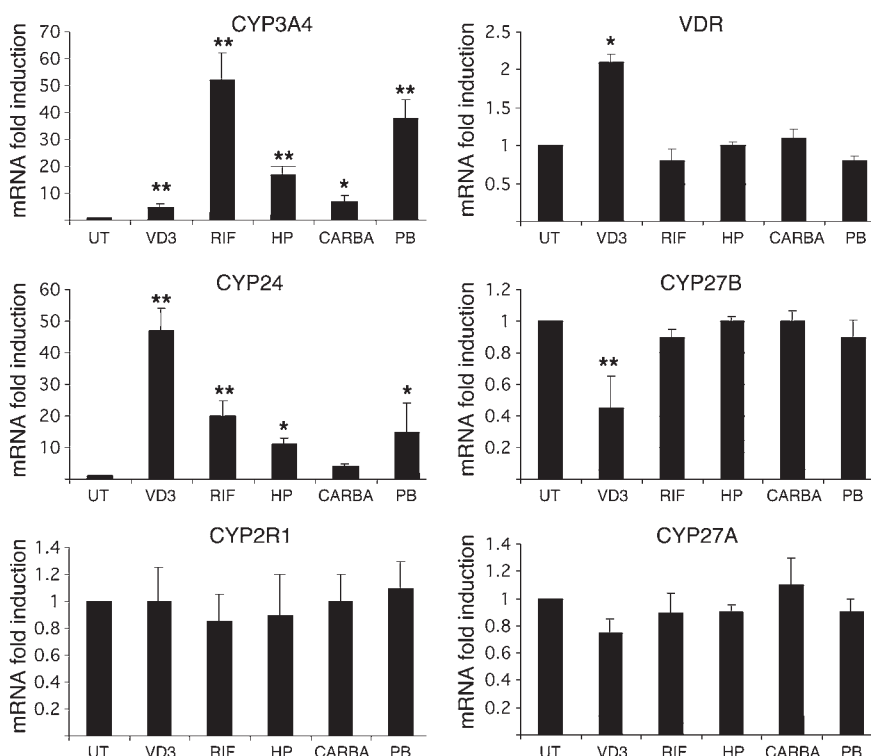


Figure 1

Effect of $1\alpha,25(\text{OH})_2\text{D}_3$, rifampicin, hyperforin, carbamazepine, and phenobarbital on CYP24, CYP2R1, CYP27B, CYP27A, VDR, and CYP3A4 mRNAs in human hepatocytes. Human hepatocytes were cultured for 48 hours in the absence (UT) or presence of the indicated compounds: 50 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (VD3), 20 μM rifampicin (RIF), 2 μM hyperforin (HP), 20 μM carbamazepine (CARBA), or 500 μM phenobarbital (PB). Total RNA was isolated using TRIzol reagent. One microgram of total RNA was reverse-transcribed, and CYP3A4, CYP24, CYP2R1, CYP27B, CYP27A, VDR, and GAPDH mRNAs were quantified by real-time RT-PCR analysis using the LightCycler apparatus (Roche Diagnostics Corp.). Data presented are means \pm SE (from 5 different cultures from 5 different liver donors) of the ratio of mRNA levels in treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels, which themselves exhibited no significant variation. Statistically significant inductions compared with those in untreated cells are marked with asterisks: * $P < 0.05$ and ** $P < 0.01$.

and carbamazepine (13–17). Indeed, long-term antiepileptic drug therapy, such as phenobarbital or carbamazepine, is a known risk factor for bone loss and fractures (13). In addition, prolonged therapy with rifampicin can cause vitamin D deficiency or osteomalacia (18), particularly in patients with marginal vitamin D stores (19). Notably, 2 weeks of rifampicin treatment reduces the levels of circulating 25-hydroxyvitamin D and $1\alpha,25$ -dihydroxyvitamin D in healthy subjects (20). The molecular mechanism of this drug-induced osteomalacia or vitamin deficiency is unknown. However, one interesting feature is that rifampicin (21), phenobarbital (22), phenytoin (23), and carbamazepine (24) are inducers of the hepatic cytochrome P450s (CYPs) involved in drug metabolism, i.e., CYP2B and CYP3A. These observations suggested to us that the orphan nuclear receptor pregnane X receptor (PXR; NR1I2 [ref. 25]) might be involved in this phenomenon. PXR has been shown to mediate CYP2 and CYP3 gene induction (25, 26) and is activated by a wide variety of commonly used drugs, notably rifampicin (25) and phenobarbital (25) but also carbamazepine (27) and phenytoin (27).

In close similarity to VDR and the enzymes involved in vitamin D biosynthesis and metabolism, PXR is expressed in intestine, kidney, and liver (25, 28), and both receptors share around 60% similarity of amino acids in their DNA-binding domains. Moreover, even though their ligand-binding domains share only 37% identity, lithocholic acid and derivatives act as ligands for both PXR and VDR (29). PXR interacts with its cognate response elements in the 5'-flanking regions of target genes by forming heterodimers with RXR α . Typically, these elements contain 2 copies of the AG(G/T)TCA hexanucleotide organized as a direct repeat with a 3- to 4-bp spacer (DR3, DR4), or an everted repeat separated by 6 bp (ER6) (30). Some of these elements are also recognized by VDR after heterodimerization with RXR, as shown with iNOS (31), CYP3A4 (32, 33), CYP2B6, and CYP2C9 (8). These observations suggest to us that these receptors are capable of

regulating common genes through the same *cis*-acting elements. This could represent an example of functional nuclear receptor cross-talk, suggesting that PXR activators might interfere with VDR-controlled physiological processes. Here, we test this hypothesis and provide data suggesting that PXR is involved in drug-induced osteomalacia by upregulating CYP24, the major enzyme responsible for the biological catabolism of vitamin D hormone.

Results

Induction of CYP24 gene expression and CYP24 activity by PXR activators in human hepatocytes. We first investigated the effects of PXR activators on the expression of key proteins involved in vitamin D₃ homeostasis, i.e., the mitochondrial 25-hydroxylase CYP27A, the microsomal 25-hydroxylase CYP2R1, the 1α -hydroxylase CYP27B, the 24-hydroxylase CYP24, and VDR. Forty-eight hours after plating, human hepatocytes were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ (50 nM) or PXR activators such as rifampicin (10 μM), hyperforin (2 μM), carbamazepine (20 μM), and phenobarbital (500 μM) for 48 hours. CYP24, CYP2R1, VDR, CYP27B, and CYP27A mRNAs were then analyzed by real-time quantitative RT-PCR using the LightCycler apparatus (Roche Diagnostics Corp.), in parallel with CYP3A4 mRNA as a control for PXR activation. We evaluated the induction ratios (mRNA levels in treated cells compared with control cells after normalization with respect to GAPDH signal) obtained from the analysis of 5 different cultures from 5 different liver donors. GAPDH and β -actin mRNA levels (data not shown) were used as quality controls of RNA preparations and RT reactions. As expected, vehicle-treated cells expressed very low levels of CYP24 (cycle threshold [Ct] = 37 ± 2 cycles), VDR (Ct = 32 ± 4), and CYP27B (Ct = 32 ± 2) mRNAs compared with CYP27A (Ct = 24 ± 1), CYP3A4 (Ct = 25 ± 5), CYP2R1 (Ct = 26 ± 2), and GAPDH (Ct = 17 ± 2).



As shown in Figure 1, expression of CYP3A4 mRNA was significantly induced by all of the compounds tested, although more modestly by $1\alpha,25(\text{OH})_2\text{D}_3$ (5-fold induction). In addition, expression of CYP24 mRNA was strongly induced by $1\alpha,25(\text{OH})_2\text{D}_3$ (47-fold induction). Interestingly, expression of CYP24 mRNA was significantly ($P < 0.05$) increased by rifampicin (20-fold induction), hyperforin (11-fold induction), and phenobarbital (12-fold induction). Carbamazepine was a weak, nonsignificant ($P > 0.07$) inducer of CYP24. The relative induction potency of rifampicin, hyperforin, carbamazepine, and phenobarbital was identical for CYP3A4 and CYP24 genes: i.e., rifampicin > phenobarbital > hyperforin > carbamazepine. In contrast, $1\alpha,25(\text{OH})_2\text{D}_3$ was a weak inducer of CYP3A4 compared with rifampicin or the other PXR activators but appeared as the most potent inducer of the CYP24 gene. As previously observed in other tissues (34), $1\alpha,25(\text{OH})_2\text{D}_3$ produced a significant decrease (50% inhibition, $P < 0.01$) of the CYP27B1 mRNA level (Figure 1). In contrast, rifampicin, hyperforin, or phenobarbital did not affect the expression of this mRNA. The level of VDR mRNA in human hepatocytes was not affected by the drugs used, except by $1\alpha,25(\text{OH})_2\text{D}_3$ (2.2-fold increase). The levels of mRNA encoding 2 enzymes involved in the 25-hydroxylation of vitamin D_3 (i.e., the mitochondrial CYP27A and the recently “de-orphanized” microsomal CYP2R1) were not affected by any of the compounds tested, even by $1\alpha,25(\text{OH})_2\text{D}_3$.

Next, we compared the extent of mRNA induction of the main PXR target gene, CYP3A4, to the extent of mRNA induction of CYP24 at various time points (from 6 to 48 hours with 20 μM rifampicin; Figure 2A), and with increasing concentrations of rifampicin (from 1 to 20 μM after 48 hours of treatment; Figure 2B). As shown, both the time-course and the dose-response profiles of induction were similar for CYP3A4 and CYP24 mRNAs. To determine whether xenobiotics induce CYP24 mRNA directly or indirectly, human hepatocytes were treated with rifampicin in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX) for 24 hours, and RNA was analyzed by semiquantitative RT-PCR using primers for CYP24 and GAPDH as control. As shown in Figure 2C, the rifampicin-mediated induction of CYP24 mRNA was not affected by CHX, although the latter compound alone increased the expression of CYP24 mRNA. It is likely that this results from an mRNA stabilization effect of this compound. In contrast, the GAPDH mRNA level was not affected by these treatments.

To investigate whether CYP24 mRNA accumulation coincided with an increase in the rate of 24-hydroxylation of vitamin D_3 , the metabolism of ^3H -25(OH) D_3 was evaluated. For this purpose, human hepatocytes were treated with $1\alpha,25(\text{OH})_2\text{D}_3$ (50 nM) or 20 μM rifampicin for 72 hours, and then incubated for 4 hours with ^3H -25(OH) D_3 . A significant increase of 24,25(OH) D_3 production was observed both after $1\alpha,25(\text{OH})_2\text{D}_3$ treatment (3.79-fold induction, $P < 0.001$) and after rifampicin treatment (1.58-fold induction, $P < 0.02$) (Table 1). The potency of these compounds to increase the rate of 24-hydroxylation of 25(OH) D_3 parallels their ability to induce CYP24 mRNA accumulation [i.e., $1\alpha,25(\text{OH})_2\text{D}_3$ > rifampicin].

In vivo induction of mouse CYP24 mRNA and 24,25(OH) D_3 plasma concentration by PXR agonists. To determine whether activators of PXR induce CYP24 in vivo, mice ($n = 5$) were treated for 6 days with dexamethasone or pregnenolone 16 α -carbonitrile, 2 well-known murine PXR agonists (28), or corn oil plus 5% DMSO (vehicle). Total RNA was prepared from liver and kidney and analyzed for cyp3a11,

cyp24, and cyclophilin (internal control) gene expression by real-time PCR. As expected, the cyp3a11 mRNA level was significantly ($P < 0.01$) and strongly increased by dexamethasone (16.3-fold) and pregnenolone 16 α -carbonitrile (9.7-fold) in the liver of treated mice in comparison to the level in vehicle-treated mice (Figure 3A). More interestingly, dexamethasone and pregnenolone 16 α -carbonitrile provoked a significant increase of cyp24 mRNA expression in kidney, 4.6-fold ($P = 0.0005$) and 2.2-fold ($P = 0.0019$) induction, respectively (Figure 3B). In addition, we measured changes in the plasma concentration of vitamin D metabolites [24,25(OH) D_3 and 25(OH) D_3] after 1 week of pregnenolone 16 α -carbonitrile treatment. As shown in Figure 3C, pregnenolone 16 α -carbonitrile provoked a significant increase of 24,25(OH) D_3 plasma level (2.01-fold induction, $P < 0.03$), while no significant effect was observed on 25(OH) D_3 circulating level (Figure 3D).

PXR transactivates human CYP24 gene promoter in Hek293 cells. The results presented above suggest that PXR is involved in the regula-

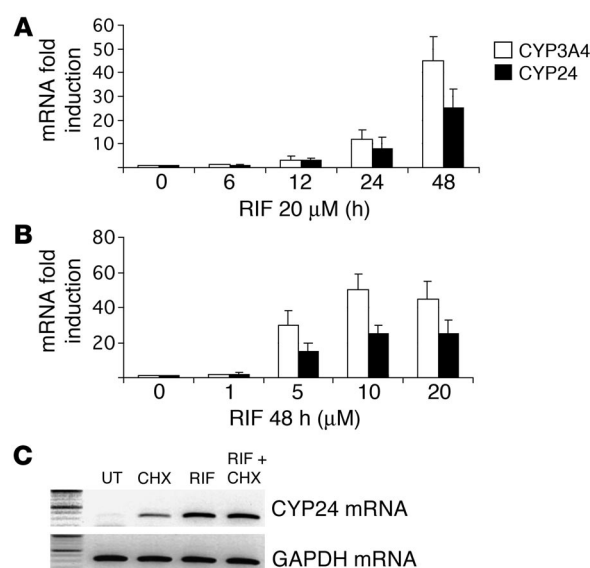


Figure 2

Effects of rifampicin on CYP24 gene transcription in human hepatocytes. (A) Time course of induction of CYP24 and CYP3A4 by rifampicin (RIF). Hepatocytes were cultured for the indicated times with or without 20 μM rifampicin. Total RNA was reverse-transcribed and analyzed by quantitative RT-PCR using primers for CYP3A4 (white bars), CYP24 (black bars), and GAPDH. Data presented are means \pm SE (from 3 different cultures from 3 different liver donors) of the ratio of mRNA levels in treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels. (B) Dose-dependent induction of CYP24 and CYP3A4 by rifampicin. Hepatocytes were cultured for 48 hours in the absence or presence of increasing concentrations of rifampicin, from 1 μM to 20 μM . Total RNA was extracted and analyzed by quantitative RT-PCR for CYP24 (black bars), CYP3A4 (white bars), and GAPDH mRNA content. Data presented are means \pm SE (from 3 different cultures from 3 different liver donors) of the ratio of mRNA levels in treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels. (C) Rifampicin has direct transcriptional effects on CYP24. Hepatocytes from liver donor number 220 were untreated (UT) or pretreated with 10 $\mu\text{g}/\text{ml}$ CHX for 1 hour before addition of 20 μM rifampicin. Total RNA was harvested 24 hours later, reverse-transcribed, and analyzed by semiquantitative RT-PCR for CYP24 (45 cycles) and GAPDH (25 cycles) mRNA content.



Table 1
CYP24 activity in human hepatocytes

Treatments	UT (n = 11)	RIF (n = 12)	VD3 (n = 12)
24,25(OH) ₂ D ₃ (fmol/well/240 min)	34.4 ± 4.3	54.0 ± 3.0 ^A	130.5 ± 11.9 ^B

Human hepatocytes (from 4 different cultures from 4 different liver donors) were cultured in the absence (UT) or presence of 50 nM 1 α ,25(OH)₂D₃ (VD3) or 20 μ M rifampicin (RIF) for 72 hours, and then incubated for 4 hours with 1.6 nM ³H-25(OH)D₃. The 24-hydroxylase enzyme activity was calculated as described in Methods. Values represent the mean \pm SE. For each experiment, 2–3 determinations were performed. ^AP < 0.02, ^BP < 0.001 versus untreated cultures incubated in the same conditions.

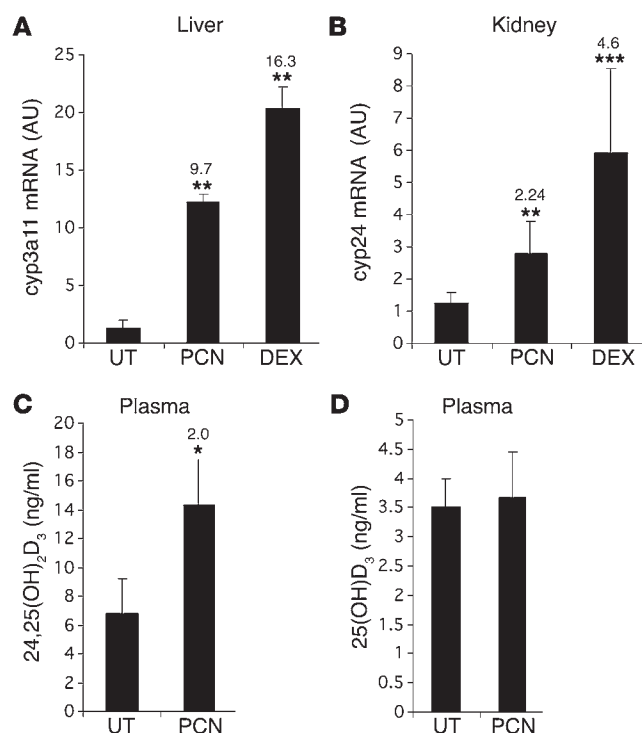
tion of the CYP24 gene. To further document this point, we first established a stable human embryonic kidney cell line (Hek293) expressing the human PXR protein. For this purpose, Hek293 cells were transfected with pIRES-hPXR/neo expression plasmid and cultured 3 weeks in a selection medium containing 600 μ g/ μ l G418. Several colonies were amplified and tested for their ability to express PXR protein, and 1 of them was used for subsequent studies. As shown in Figure 4A, this cell line (Hek293-hPXR) expresses human PXR protein, as assessed by Western blotting performed with an anti-human PXR serum, while the parental Hek293 cells do not. Moreover, in contrast to the parental cells, these cells respond to rifampicin, as observed in transient transfection assays using a CYP2B6 (NR1B3)-tkLUC reporter construct, which contains 3 repeats of a functional PXR-responsive element of the CYP2B6 gene (26) (Figure 4B). We then analyzed the effect of 1 α ,25(OH)₂D₃ (50 nM), rifampicin (10 μ M), or hyperforin (2 μ M) on the endogenous CYP24 mRNA level in both parental and PXR-expressing Hek293 cell lines. As shown in Figure 4C, treatment with 1 α ,25(OH)₂D₃ for 48 hours produced 9- and 7-fold inductions of CYP24 mRNA in the parental cells (reflecting the endogenous expression of VDR) and in Hek293-hPXR cells, respectively. In contrast, while rifampicin and hyperforin had no effect on CYP24 mRNA expression in control cells, these compounds produced 4.5- and 3-fold increases of CYP24 mRNA in the Hek293-hPXR cells, respectively.

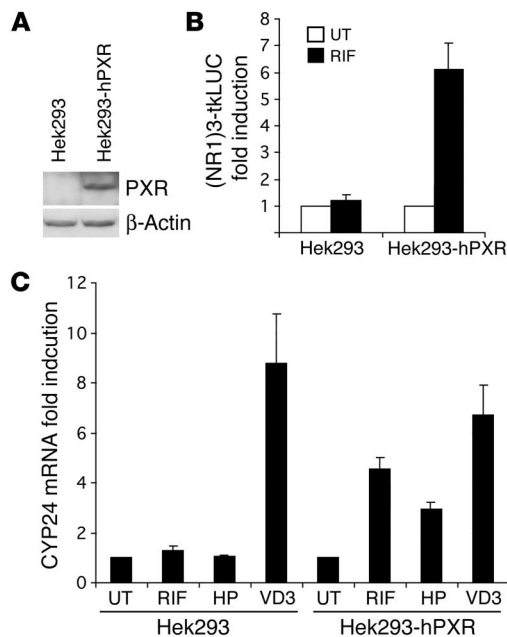
Figure 3

In vivo modulation of CYP24 by PXR agonists. (A and B) Effect of pregnenolone 16 α -carbonitrile and dexamethasone on cyp24 mRNA abundance. Mice (n = 5) were injected i.p. for 6 consecutive days with dexamethasone (DEX; 10 mg/kg/d), pregnenolone 16 α -carbonitrile (PCN; 100 mg/kg), or corn oil (UT). Total RNA from liver or kidney was prepared, and 1 μ g was reverse-transcribed. The relative levels of cyp3a11 and cyp24 mRNAs were determined in duplicate for each mouse by real-time PCR using cyp3a11-specific (A) and cyp24-specific (B) primers. Cyclophilin mRNA levels were used as a reference standard. Data are means \pm SE of the ratio of mRNA levels in treated mice to corresponding levels in untreated mice, normalized with respect to cyclophilin mRNA levels. (C and D) Effect of pregnenolone 16 α -carbonitrile on vitamin D₃ metabolites in mouse plasma. Mice (n = 5) were injected i.p. for 6 consecutive days with pregnenolone 16 α -carbonitrile (100 mg/kg) or corn oil and plasma samples. Pooled mouse plasma (1–2 mice, 300 μ l, n = 3) or 50- μ l plasma samples (n = 5) were analyzed for 24,25(OH)₂D₃ (C) or 25(OH)D₃ metabolites (D), respectively, as described in Methods. Statistically significant expressions compared with untreated mice are marked with asterisks: *P < 0.05, **P < 0.01, and ***P < 0.005. Fold change relative to control mice is indicated.

The cloning of the human CYP24 promoter has been reported (35). To determine the nature of the *cis*-acting elements involved in PXR-mediated CYP24 gene expression, analysis of promoter transcriptional activity was undertaken. Various deletions of the human CYP24 promoter were constructed into a promoterless luciferase reporter vector (pGL3basic; Figure 5A). These constructs were tested for their ability to respond to PXR by transient cotransfection assays in Hek293 cells. In response to rifampicin, the luciferase activity of the 1.2-kb promoter fragment (–1,200 to –22) exhibited a 4-fold increase under cotransfection with human PXR cDNA expression vector (Figure 5B). The magnitude of the response was identical to that observed with the –326 to –22 construct. However, the –144 to –22 construct did not respond to rifampicin. Similar results were obtained with pregnenolone 16 α -carbonitrile and a mouse PXR expression vector. Therefore, the –326 to –144 region of the CYP24 promoter confers response to both human and murine PXR. Interestingly, this region is responsive to 1 α ,25(OH)₂D₃ even in the absence of cotransfection of the receptor expression vector (Figure 5B), because Hek293 cells express endogenous VDR (36). Indeed, 2 VDREs have been defined within the –300 to –150 region of the human and mouse CYP24 promoter that confer response of the promoter to both 1 α ,25(OH)₂D₃ (35) and RXR ligands (37).

PXR binds to and transactivates the VDRE-I and VDRE-II motifs of human CYP24 promoter. We have previously reported that VDR binds to and transactivates PXR-responsive elements present on the promoter of several CYP genes, including CYP2C9, CYP2B6, and CYP3A4. This suggested that these receptors could share DNA-responsive elements and target genes (8). The –316 to –144 region of the human CYP24 promoter contains 3 potential binding sites for PXR: 2 VDREs previously identified, –294 to –274 (VDRE-II) and –174 to –151 (VDRE-I), and the –316 to –291 region (CYP24 site 3), which contains sequences that are homologous with various



**Figure 4**

Rifampicin induces the CYP24 gene in a Hek293 cell line stably transfected with PXR. **(A)** Expression of human PXR in parental and pIRES-hPXR/neo-transfected Hek293 cells. Whole-cell extracts were prepared from parental Hek293 cells or pIRES-hPXR/neo-transfected cells (Hek293-hPXR). Proteins were separated on 10% SDS-PAGE, and PXR and β -actin expression was monitored by Western blotting (Santa Cruz Biotechnology Inc.). **(B)** Rifampicin activates an NR1-LUC reporter gene in Hek293-hPXR cells. Parental Hek293 or Hek293-hPXR cell lines were transiently transfected with CYP2B6 NR1-LUC reporter gene together with pRSV- β -gal transfection control plasmid. After transfection, cells were treated with 0.1% DMSO (UT; white bars) or 10 μ M rifampicin (RIF; black bars). Values represent β -gal-corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates \pm SE. These were replicated in independent experiments. **(C)** Rifampicin and hyperforin induce the CYP24 gene in Hek293-hPXR cells. Parental Hek293 or Hek293-hPXR cell lines were untreated (UT) or treated with 50 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (VD3), 10 μ M rifampicin (RIF), or 2 μ M hyperforin (HP) in triplicate. Forty-eight hours later, total RNA was extracted and analyzed by quantitative RT-PCR for CYP24 and GAPDH mRNA content. Values represent the average \pm SE.

repeat motifs (Figure 6A). The ability of these sequences to bind to the PXR:RXR α heterodimer was tested by competition in electrophoretic mobility shift assays using the proximal PXR-responsive element of CYP3A4 (ER6 motif [ref. 25]). PXR and RXR α were prepared by *in vitro* translation of their cDNAs. As expected, PXR:RXR α heterodimer produced a retarded band with the CYP3A4 ER6 probe (Figure 6B, lane 1), which was decreased by an excess of ER6 unlabeled probe (lanes 3 and 4). Interestingly, the 2 CYP24 VDREs produced a dose-dependent decrease of PXR:RXR α binding to the CYP3A4 ER6 probe (Figure 6B, lanes 5–8), which suggests direct competition between the CYP3A4 ER6 and VDRE motifs in the interaction with PXR:RXR α heterodimers. In contrast, the CYP24 –316 to –291 region (site 3) failed to compete with the CYP3A4 ER6 probe (Figure 6B, lane 2), which suggests that this element is not required for PXR-mediated CYP24 gene induction.

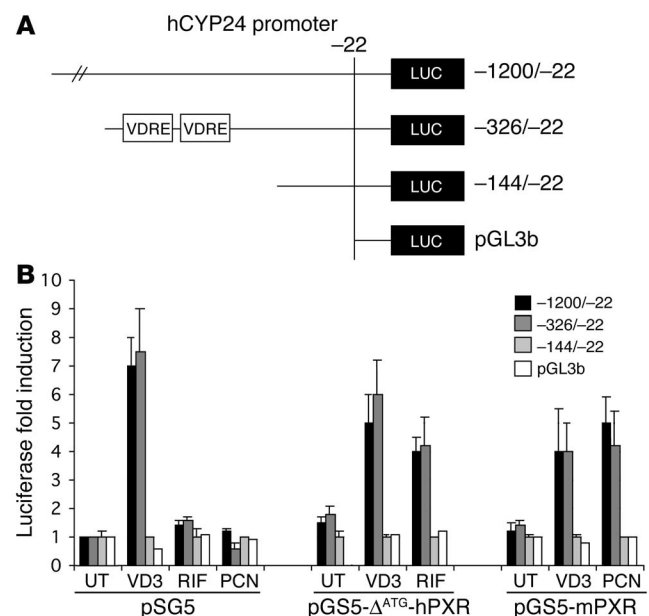
To test the direct interaction between PXR and the 2 VDREs of the CYP24 promoter, oligonucleotides VDRE-I (Figure 7, lanes 1–6) and VDRE-II (lanes 7–12) were end-labeled and subjected to electrophoretic mobility shift assay. Note that the reticulocyte lysate produced a nonspecific retarded band (Figure 7, NS). Nevertheless, *in vitro*-synthesized PXR:RXR α and VDR:RXR α

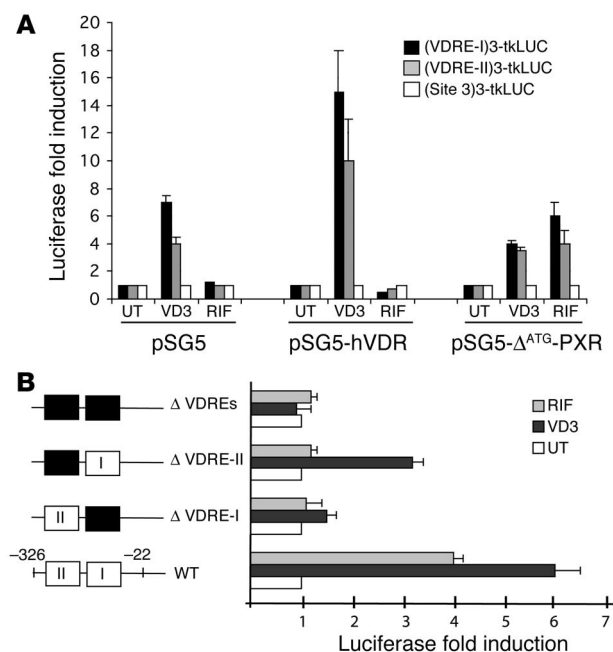
heterodimers efficiently bound to the 2 CYP24 VDRE motifs (Figure 7, lanes 5, 6, 11, and 12). This binding was observed only in the presence of the heterodimerization partner RXR α , while neither PXR, RXR α , nor VDR alone bound to these sequences. These results strongly suggest that the PXR:RXR α heterodimer binds to the 2 VDREs of the human CYP24 promoter.

To demonstrate further the role of CYP24 VDRE-I and VDRE-II motifs in PXR-mediated CYP24 gene induction, we used heterologous promoter constructs containing 3 repeats of CYP24 site 3 (–316 to –291), VDRE-I, or VDRE-II elements upstream of the minimal thymidine kinase promoter (tkLUC). Transient cotransfection assays were performed on Hek293 cells with these reporter plasmids and VDR or PXR cDNA expression vectors (Figure 8A). As expected, both VDRE-I and VDRE-II reporter

Figure 5

PXR transactivates the proximal human CYP24 promoter. **(A)** VDREs present in the human CYP24 (hCYP24) gene. Schematic representation of the human CYP24 constructs used in this work. **(B)** Ligand-activated PXR transactivates the –326 to –143 promoter region of human CYP24. Hek293 cells were transiently transfected with human PXR (pSG5- Δ^{ATG} -hPXR), mouse PXR (pSG5-mPXR), or control expression vector (pSG5) in the presence of reporter vectors driven by the indicated human CYP24 promoter sequences, together with pRSV- β -gal transfection control plasmid. Cells were treated with vehicle (0.1% DMSO; UT), 50 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (VD3), 10 μ M rifampicin (RIF), or 10 μ M pregnenolone 16 α -carbonitrile (PCN) for 24 hours. Cells were then harvested and analyzed for both luciferase and β -gal activities. Values represent β -gal-corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates \pm SE. These were replicated in independent experiments.



**Figure 8**

Functional and mutational analysis of human CYP24 VDREs. (A) Thymidine kinase promoter luciferase reporter vectors (pGL3tkLUC) driven by 3 repeats of oligonucleotides from the human CYP24 promoter (site 3, VDRE-I, and VDRE-II) were cotransfected with control expression plasmid (pSG5), human VDR (pSG5-hVDR), or human PXR (pSG5-Δ^{ATG}-hPXR) into Hek293 cells, together with pRSV-β-gal transfection control plasmid. Cells were treated with vehicle (0.1% DMSO; UT), 50 nM 1α,25(OH)₂D₃ (VD3), or 10 μM rifampicin (RIF) for 24 hours. Cells were then harvested and analyzed for both luciferase and β-gal activities. Values represent β-gal-corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates ± SE. They were replicated in independent experiments. (B) Hek293 cells were transiently cotransfected with pSG5-Δ^{ATG}-hPXR expression vector along with various human CYP24 promoter sequence-containing reporter constructs, together with pRSV-β-gal transfection control plasmid. Cells were treated with vehicle, 50 nM 1α,25(OH)₂D₃, or 10 μM rifampicin for 24 hours. Reporter constructs were as follows: -316 to -22 wild-type sequence in pGL3b (WT) or point mutants of VDRE-I (-168CCC mutated to GTT; ΔVDRE-I), VDRE-II (-289CACC to AAAA; ΔVDRE-II), or both VDREs (ΔVDREs). Values represent β-gal-corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates ± SE. They were replicated in independent experiments.

a multifunctional enzyme that directs the side-chain oxidation and cleavage of 25(OH)D₃ and 1,25(OH)₂D₃ to catabolic carboxylic acid end products (43). It is well established that induction of the rat CYP24 gene by 1,25(OH)₂D₃ is mediated by 2 VDREs located about 100 bp apart within the 300-bp proximal promoter (35). In human hepatocytes, PXR-mediated CYP24 mRNA accumulation was not inhibited by CHX pretreatment, which suggests a direct effect of PXR on CYP24 gene expression. In addition, transfection studies in human embryonic kidney cells demonstrated that both human PXR and mouse PXR directly transactivate the CYP24 promoter. Moreover, we found that PXR binds to and transactivates the 2 proximal VDREs present in the CYP24 promoter. Point mutations within the 5' half-site of either the upstream VDRE (ΔVDRE-II) or the downstream VDRE (ΔVDRE-I) led to a total inhibition of PXR-mediated induction of luciferase activity, which suggests that both sites are required for an efficient PXR-mediated CYP24 gene expression. Such cooperation between several PXREs is frequently observed within PXR target genes. In summary, our results demonstrate that CYP24 is a VDR target gene as well as a PXR target gene. Moreover, we observed that, in vivo in mice, activation of PXR by pregnenolone 16α-carbonitrile significantly increased the circulating level of 24,25(OH)₂D₃ (2-fold induction). These results fully prove our hypothesis and are in agreement with those previously obtained by others. Indeed, pharmacological doses of dexamethasone, another mouse PXR agonist and cyp3a inducer (28), increase intestinal and renal cyp24 mRNA accumulation and enzyme activity (44).

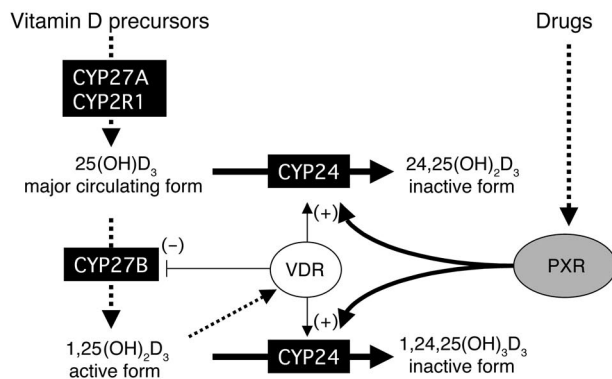
Since 24-hydroxylation is the initial enzymatic step in the catabolism of 1,25(OH)₂D₃, increased cyp24 gene expression and 24(OH)-ase activity might be expected to result in lower cellular concentrations of 1,25(OH)₂D₃ and, therefore, lower expression of VDR target genes (Figure 9). Indeed, many reports show that drugs now identified as PXR activators produce vitamin D deficiency and increase the risk of bone fracture. It is well-known that long term treatment with phenytoin, phenobarbital, rifampicin, or carbamazepine impairs bioavailability of vitamin D and bone mineralization, leading to osteomalacia and osteoporosis

(13, 15–18, 20, 45–47). Consistent with this, it has been observed that the circulating level of 25(OH)D₃ or 1,25(OH)₂D₃ is decreased by these drugs (18, 20, 48, 49). In addition, osteopenia, osteoporosis, and osteonecrosis are the most significant bone disorders affecting patients suffering from HIV infection (50), and HIV-infected patients receiving antiretroviral therapy have a high prevalence of reduced bone mineral density (51). The mechanisms of these phenomena are complex and unclear. However, a part of these effects might be the consequence of CYP24 gene upregulation induced by the activation of PXR. Indeed, protease inhibitors have been suspected to play some role in this process (52, 53), and 2 of them, ritonavir and saquinavir (27, 40), are PXR agonists.

In the most classical view of molecular toxicology, undesirable effects of xenobiotics are primarily ascribed to the chemical reactivity of the parent compounds and/or of some of their metabolites that are produced during oxidative metabolism mediated notably by CYPs (e.g., covalent binding to proteins and DNA, effects on membrane lipids, etc.). A second aspect of the undesirable effects of xenobiotics results from the fact that these compounds may affect the level and activity of xenobiotic detoxification systems (interactions, induction, and inhibition). In this respect, although PXR evolved to protect the body from xenobiotic-mediated toxic effects, its activation by some drugs represents the basis for a common class of potentially life-threatening drug interactions in which one drug accelerates the metabolism of another (54). The cross-talk described here between PXR and VDR represents another aspect of the undesirable effects of xenobiotics, in which a xenobiotic-mediated increase in endobiotic-compound metabolism/catabolism can lead to endocrine system perturbation.

Methods

Chemicals. DMEM, DMSO, dexamethasone, rifampicin, 1α,25(OH)₂D₃, carbamazepine, hyperforin, pregnenolone 16α-carbonitrile, and culture medium additives were from Sigma-Aldrich. The radioisotope γ-[³²P]d-ATP was from Amersham International. Gelatin was purchased from Touzart-Matignon, neutral charcoal from NORIT, and dextran 20 from OSI

**Figure 9**

Proposed model for PXR-mediated drug-induced osteopenia or osteomalacia. While $1\alpha,25(\text{OH})_2\text{D}_3$ maintains vitamin D homeostasis via downregulation of vitamin D_3 biosynthesis enzymes (CYP27B) and upregulation of the vitamin D -inactivating enzyme (CYP24), drugs that activate PXR may be responsible for the acceleration of vitamin D catabolism through the upregulation of CYP24, leading to vitamin D deficiency and eventually to osteopenia or osteomalacia.

Pharmaceuticals. Unlabeled $25(\text{OH})\text{D}_3$ was a gift from Laboratoires Roussel-Uclaf, $24,25(\text{OH})_2\text{D}_3$ was a gift from M.L. Uskokovic (F. Hoffmann-La Roche Ltd.), and $26,27\text{-}^3\text{H}$ -dihydroxyvitamin D_3 (specific activity, 23 Ci/mmol) was purchased from Amersham Biosciences Europe GmbH. Chromatography solvents (n-hexane and isopropanol), methanol, and chloroform were purchased from Merck SA.

Plasmids. The following plasmids have been described previously: pSG5- Δ^{ATG} -hPXR (28), pSG5-mPXR (28), pSG5-mRXR α (8), and pSG5-hVDR (8). pIRES-hPXR/neo plasmid was generated by PCR amplification (High-Fidelity PCR system; F. Hoffmann-La Roche Ltd.) of cDNA encoding amino acids 1–434 of human PXR using oligonucleotides 5'-GGGTGTGGGGAATTCACCACCATGGAGGTGAGACCCAAAGAAAGC and 5'-GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG and insertion into pIRESneo (BD Biosciences – Clontech) digested with EcoRI/BamHI.

Human CYP24 promoter deletions were obtained by PCR (*Pfu* polymerase; Promega Corp.) using CYP24 forward 18-mer oligonucleotide primers (–1,200 to –1,182, –326 to –308, or –144 to –126) with an overhanging SalI site immediately upstream of the 5'-most base of the deletion construct; a common 3' primer, TGCATTTATGGAGACAGA (CYP24 –39 to –22); and the human CYP24 (–1,250 to +99) pBLCAT3 plasmid as a template (a gift from H.F. DeLuca, University of Wisconsin – Madison, Madison, Wisconsin, USA). PCR products were then digested by SacI and subcloned in SacI/SmaI-linearized pGL3basic vector (Promega Corp.) to produce –1,200-LUC, –326-LUC, and –144-LUC constructs.

pGL3tkLUC plasmid was obtained by ligation of the HindIII/BglII tk-SEAP vector fragment (BD Biosciences – Clontech), corresponding to the minimal 147nt long tk promoter, into the HindIII/BglII-linearized pGL3basic vector. Oligonucleotides corresponding to the individual CYP2B6 NR1 (55), CYP2A4 site 3 (–316 to –291), and VDREs (VDRE-II, –294 to –274, and VDRE-I, –174 to –151) (37) were synthesized with BamHI and BglII ends, annealed, and ligated, and 3 copies were subcloned into the BamHI/BglII-digested pGL3tk. The resulting (NR1)3-tkLUC, (site 3)3-tkLUC, (VDRE-II)3-tkLUC, and (VDRE-I)3-tkLUC were verified by sequencing.

The specific mutations of the VDREs in the native CYP24 promoter (–326 to –22) were introduced by PCR-based site-directed mutagenesis (QuikChange PCR-mediated site-directed mutagenesis kit; Stratagene) using –326-LUC vector as a template to produce $\Delta\text{VDRE-I}$ (–168CCC

mutated to GTT) and $\Delta\text{VDRE-II}$ (–289CACC mutated to AAAA). The $\Delta\text{VDRE-II}$ vector was then used as a template to produce the $\Delta\text{VDRE-I} + \Delta\text{VDRE-II}$ (ΔVDREs) construct.

Primary culture of human hepatocytes. Hepatocytes were prepared from lobectomy segments resected from adult patients for medically required purposes unrelated to our research program. The use of these human hepatic specimens for scientific purposes has been approved by the French National Ethics Committee (Paris, France). Hepatocytes were prepared and cultured according to a previously published procedure (56). The cells were plated into collagen-coated dishes at 0.17×10^6 cells per square centimeter in a hormonally and chemically defined medium consisting of a mixture of Williams's E medium and Ham's F12 medium (1:1 in volume). Forty-eight hours after plating, cells were cultured in the presence of the indicated concentrations of compounds.

Quantitative PCR. Total RNA was extracted from cells or frozen mouse tissues using TRIZOL reagent (Invitrogen Corp.), according to the manufacturer's instructions. Purity was confirmed by spectrophotometry. cDNAs were synthesized from 1–2 μg of total RNA using the SuperScript II first-strand synthesis system for PCR (Invitrogen Corp.) at 42°C for 60 minutes, in the presence of random hexamers (Amersham Pharmacia Biotech). Quantification of mRNAs was performed using the LightCycler apparatus (Roche Diagnostics Corp.). Two microliters of diluted RT reaction (1:10) was used for real-time PCR amplification. The following program was used: a denaturation step at 95°C for 8 minutes, and 50 cycles of PCR (denaturation, 95°C, 15 seconds; annealing, 67°C, 10 seconds; extension, 72°C, 20 seconds). In all cases, the quality of the PCR product was assessed by monitoring of a fusion step at the end of the run. Mouse sense and reverse primers, respectively, were as follows: *cyp24a1*, 5'-CTCCCTATGGATGCAGTATGTATAGTG and 5'-TTTAAAACGTTGTCAGTAGGTCATAACT; *cyp3a11*, 5'-GAAGGAAAGCCGCTGGATT and 5'-GGGTGAGTGGCCAAGGAATG; cyclophilin, 5'-TGGAGAGCACCAAGACAGACA and 5'-TGCCGGAGTCGACAATGAT. Human sense and reverse primers, respectively, were as follows: CYP3A4, 5'-CACAAACCGGAGGCCTTTTG and 5'-ATCCATGCTGTAGGCCCCAA; GAPDH, 5'-GGTCGGAGTCAACGGATTGGTTCG and 5'-CAAAGTTGTCATGATGACC; CYP24 (NM_000782), 5'-GTGGCTCCAGCCAGACCCTA and 5'-GGCGAGGTTGGTACGAGGTG; CYP27B (NM_000785), 5'-CGAGAAGGACCTGGAGTCTG and 5'-TCTGGACACGAGAATTTC; CYP27A (NM_000784), 5'-GCTTCTCTTCCCAAGAAC and 5'-ACTGCAGGCCCACTTTCTTA; VDR (NM_000376), 5'-AGCAGCGCATCATTGCCATA and 5'-CAGCATGGAGAGCTGGGACA. Human and mouse CYP24, CYP2R1, CYP27B, CYP27A, and VDR mRNA amplified fragments were purified using the NucleoSpin extract kit (Macherey-Nagel Co.) and verified by sequencing.

$25(\text{OH})\text{D}_3$ 24-hydroxylase assay. Human hepatocytes plated in collagen-coated dishes were treated for 72 hours with 50 nM $1,25(\text{OH})_2\text{D}_3$, 20 μM rifampicin, or 500 μM phenobarbital. After rinsing with medium, 1.6 nM ^3H - $25(\text{OH})\text{D}_3$ (0.05 μCi) were added, and the cells were incubated for an additional 4 hours in 1 ml medium. The medium was removed and the ^3H -vitamin D_3 derivatives present in the medium were extracted by addition of 2 ml methanol plus 2 ml chloroform. The chloroform extract was cochromatographed with 100 ng unlabeled $24,25(\text{OH})_2\text{D}_3$ using a straight-phase HPLC system (Beckman Coulter Inc.) equipped with a 4.6×250 mm Ultrasphere Si column (Altex; Beckman Coulter Inc.), and equilibrated with n-hexane and isopropanol (95:5) (flow rate 1.5 ml/min). Absorbance at 254 nm was continually monitored, and effluent fractions were collected every minute. After evaporation to dryness, fractions were dissolved in Ultima Gold scintillation fluid (Packard Instrument Co.), and the radioactivity was measured. The 24-hydroxylase enzyme activity (femtomoles per culture well per 240 minutes) was calculated based on the percentage of the



injected radioactivity recovered in the 24,25(OH)₂D₃ regions after HPLC. The data were subjected to an overall statistical evaluation using ANOVA. Test groups were compared with respective untreated control groups using the Mann-Whitney nonparametric *U* test.

Animals and treatments. Male mice (C57BL/6; Charles River Laboratories France) were housed in a pathogen-free animal facility under a standard cycle of 12 hours light, 12 hours dark. After 1 week of acclimatization, pregnenolone 16 α -carbonitrile (100 mg/kg/d) or dexamethasone (10 mg/kg/d) was injected in corn oil plus 5% DMSO through 6 successive daily administrations (*n* = 5 for each group). The mice were killed 4 hours after the last pregnenolone 16 α -carbonitrile administration, and plasma and tissues (liver and kidney) were snap-frozen in liquid nitrogen.

Measurements of vitamin D metabolites in mouse plasma. The 25(OH)D₃ metabolite was extracted from 50- μ l plasma samples (*n* = 5) with methanol-chloroform, and chromatographed on Amprep C18 Mini-Columns (RPN1900; Amersham Biosciences Europe GmbH) to separate 25(OH)D₃ from vitamin D (57). The 24,25(OH)₂D₃ metabolite was extracted from 300- μ l plasma samples (pool of 1–2 mice, *n* = 3) with methanol-chloroform, and chromatographed on a straight-phase HPLC system (Beckman Coulter Inc.) using 92% *n*-hexane and 8% isopropanol as solvents. The 25(OH)D₃ region from Amprep C18 chromatography and the 24,25(OH)₂D₃ region from HPLC were dried down and dissolved in ethanol. They were then assayed by the competitive protein-binding assay of Preece et al. (58), with 2 modifications: serum vitamin D-binding protein was obtained from vitamin D-fed and not from vitamin D-deficient rats, and gelatin (3 g/l) was used instead of albumin. The quality of the results was controlled by simultaneous assessment of external standards (Vitamin D External Quality Assessment Scheme [DEQAS]).

Cell culture and transfections. Hek293 cells (human embryonic kidney cells) were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen Corp.). Transfection of plasmid DNA was performed in single batches with FuGENE 6 (Roche Diagnostics Corp.) according to the manufacturer's instructions. For reporter analysis, transfections were performed using 80,000 cells, 150 ng of luciferase-based reporter plasmids, and expression plasmids for mouse PXR (50 ng of pSG5-mPXR.1), human PXR (50 ng of pSG5- Δ ^{ATG}-hPXR), or human VDR (25 ng of pSG5-hVDR). As internal control, 50 ng of pRSV- β -gal (Promega Corp.) was added. After 12–16 hours, the medium was changed, and fresh medium containing 5% delipidated charcoal-stripped serum (Sigma-Aldrich) and 0.1% DMSO (vehicle) or inducers was added. Twenty-four hours after the medium was changed, the cells were harvested in reporter lysis buffer (Promega Corp.), and cell extracts were analyzed for luciferase and β -gal activities as described previously (8). Luciferase values were normalized with β -gal values calculated by division of the maximal response by the response elicited with vehicle.

Stable Hek293 clones expressing human PXR (Hek293-hPXR) were obtained by transfection of Hek293 cells with pIRES-hPXR/neo and, after 3 weeks of selection, with 600 μ g/ml G418. Individual clones were then amplified and analyzed for PXR mRNA and protein expression levels.

Western blot analysis. Whole-cell lysates (10⁶ cells) were prepared from Hek293 or stable transfectant Hek293-hPXR cell lines, with 300 μ l of 100 mM Tris (pH 8.5), 250 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM DTT, and anti-protease tablet from Roche Diagnostics Corp. Following centrifugation (12,000 *g* for 10 minutes at 4°C), the supernatant was collected and stored at –80°C. Protein content was determined by bicinchoninic acid (BCA) protein assay (Interchim). Fifty-microgram extracts were subjected to 10% SDS-PAGE and transferred to PROTRAN nitrocellulose membrane (Schleicher & Schuell BioScience Inc.). Immunoblotting was carried out with actin antibody (I-19) and PXR antibody (N-16), both purchased from Santa Cruz Biotechnology Inc. Chemiluminescence detection was performed using HRP-conjugated secondary antibodies and the Amersham Biosciences Europe GmbH ECL kit.

Electrophoretic mobility shift assays. Double-stranded oligonucleotides corresponding to the 2 VDREs and site 3 of human CYP24 were synthesized (Invitrogen Corp.): VDRE-I (–174 to –151), CCGGACGCCCTCGCTCACCTCGCTGA; VDRE-II (–294 to –274), CGAAGCACACCCGGTGAAGTCCGG; site 3 (–316 to –291), GGAGGGCGGGGAGGCGCGTTCGAAG. The CYP3A4 ER6 probe and binding procedure have previously been described (8). Probes were end-labeled with ³²P-ATP using T4 polynucleotide kinase (Promega Corp.) and then incubated for 20 minutes at room temperature with 1 μ l of in vitro-synthesized PXR, VDR, or RXR α (transcription and translation-coupled [TNT-coupled] transcriptional translation; Promega Corp.). DNA-protein complexes were subjected to electrophoresis on a 4% polyacrylamide gel (30:1 acrylamide/bis-acrylamide) in 0.5 \times TBE (1 \times TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography with XAR film (Eastman Kodak Co. Scientific Imaging Systems).

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Address correspondence to: J.M. Pascussi, INSERM 632, IFR122, Campus CNRS, 1919, Route de Mende, 34293 Montpellier, France. Phone: 33-4-67613369; Fax: 33-4-67523681; E-mail: pascussi@montp.inserm.fr.

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